ddRAD library prep
Norah Saarman - Aug 24, 2024

OVERVIEW

1. **Make AmPure XP magnetic beads** (or check that there is a fresh batch that has been tested and is ready for use) 1
2. **Anneal adaptors** (or check that there are some annealed and ready for use) 1
3. **Stage DNA** in a plate in recorded order (need at least 50 nanograms of DNA per individual in 44 µL)
    - Standardize concentrations (Qubit/Plate-Reader → C1 x V1 = C2 x V2)
    - Calculations can be made using the TEMPLATE ddRAD library prep spreadsheet
4. **Double digest** DNA (EcoRI, MspI), clean 2
    - Magnetic beads clean-up
5. **Ligate adaptors** (make sure you know which **barcode** to use for each individual) 3
6. **PCR amplification**, duplicate 20 uL reactions
7. **Clean with AmpMagnetic beads clean-up**, combine the duplicate PCR reactions into 40 uL beforehand. 3
8. **Quantify with Qubit or Plate Reader.** 3
9. **Pool barcodes** with standardized concentrations across each set of 24 barcoded samples 3
    - Standardize concentrations (Qubit/Plate-Reader → C1 x V1 = C2 x V2)
    - Amicon Ultra 30KDA centrifugal filter columns
10. **Submit for Blue Pippin, QC, sequencing** (Blue Pippin 1.5% agarose Internal R2) 4

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BENCH PROTOCOL:

1. Make AmPure XP magnetic beads. The AmpureXP beads replacement recipe significantly cuts total costs of the protocol. See Appendix IV for details.

2. Anneal adaptors. Make a plate of adapters at working concentrations (10 µM).

- **EcoRI_P1 with barcode:** Mix 1 µL of each oligo in a pair (100 µM stock) with 98 µL of water to make 100 µL of 1 pmole/µL (1 µM) of annealed, double-stranded adaptor stock. Heat to 95◦C for 5 minutes and slowly cool to room temperature. This annealing step only needs to be performed once for a stock solution of the adapter. Keep the set of adaptors organized in plate format that is convenient for later use in setting up reactions.

- **MspI_P2:** Mix 10 µL of the MspI_P2.1 and MspI_P2.2* oligos (100 µM stock) with 80 µL of water to make 100 µL of 10 pmol/µL (10 µM) working stock. Heat to 95◦C for 5 minutes and slowly cool to room temp to anneal oligos into double-stranded adaptor. This annealing step only needs to be performed once for a stock solution of the adapter.

    NOTE: These oligos are the same for each fragment. On an Illumina sequencer and with our configuration of the sequencing primer, sequencing only occurs from the EcoRI side, so there is no need to barcode the MspI oligos.

3. Stage DNA (per library). Measure concentrations of all samples to be digested, concentrate by speed-vac when needed. Ideally, you want to digest 1000 ng or 1 µg, but you can use as little as 50 ng. Measure concentrations with Quant-it Picogreen ds reagent and plate reader.

- **Quantify DNA with Qubit or Plate-Reader (Quant-it Picogreen), see Appendix VII.**

- Use final concentrations to calculate volume of DNA to add to 44 µL, then make sure to top off to 44 µL with UltraPure H20 before proceeding.

  $C_1 * V_1 = C_2 * V_2$
  $C_1 * V_1$ = ~50-500 ng desired
  Volume to add in µL = ~50-500 ng / [concentration measured] ng/µL

4a. Double Digest. Consult product information for your chosen restriction enzymes regarding digest conditions.

- Double digest ~50-500 ng of high quality genomic DNA with selected restriction enzymes, in a 50µL reaction volume, in PCR tubes or 96-well plates:
-

X1 Double Digest		**X110** (96 + 14 slop = 110)
5 µL	New England Biolabs CutSmart Buffer	550 µL
0.8 µL	µLtraPure H2O	88 µL
0.1 µL	EcoRI enzyme	11 µL
0.1 µL	MspI enzyme	11 µL
		660 µL
(add 6µL to each sample DNA of 44 µL)		(~82 µL per tube in strip)

- Make sure to mix well and spin down before proceeding. To ensure complete digestion, we run our double digests for 3 hours at 37°C in a thermocycler. **Do not heat kill the enzymes**, as this may skew base composition in the resulting fragment library. Before proceeding with step 3, cool the reaction to room temperature. Alternatively, reactions can be stored at 4°C overnight (program “DDIGEST_37" in “SAARMAN” folder).

4b. Clean the double digest with AMPure XP beads

- Make sure the AMPure XP beads are at room temp and well mixed before starting!
- Add 75 µL AMPure XP to each 50 µL digestion (this is 1.5 X beads, can be adjusted based on the beads, volume, size targeting, etc.). <u>Pipette up & down 10 times as you are adding the beads to the well.</u>
- Incubate at room temperature for **10 min**
- Place reactions in magnetic plate for **2 min**
- Aspirate the cleared solution and discard
- Dispense 200 µL of fresh 70% ethanol to each well of the reaction plate and incubate for 30 seconds at room temperature
- Aspirate out the ethanol and discard
- Repeat for <u>last two steps</u> one more time for a total of two washes
  - Make sure you get all of the ethanol out the second time, using a smaller pipette if needed.
  - Let beads dry for 1-2 minutes
  - Making sure you don’t let it get too dry (cracking-earth look = too dry)
- Remove from the magnet plate, add 46 µL of µLtraPure H2O, pipette up and down 10 times
- Place the reactions back on the magnet and allow separation for **1 min**, transfer 44 µL to a new tube.

5a. Adapter Ligation. Make master mix (50 µL reactions) that contains 5 µL master mix with P2 adaptor and T4 ligase to each tube. You will add this plus 1 µL each barcoded annealed P1 adaptor to each sample 44 µL double digested DNA:

X1 Adaptor Ligation with T4		**X110** (96 + 14 slop = 110)
3.3 µL	µLtraPure H2O	363 µL
0.5 µL	10X T4 buffer	55 µL
0.2 µL	T4 enzyme	22 µL
1 µL	MspI_P2 adapters (annealed)	110 µL
5 µL	mastermix per tube	550 µL

(add 5 µL mm per tube, plus 1 µL EcoRI_P1 with barcoded annealed adaptor to each sample)

(~68.5 µL mm per tube in strip of 8 for pipetting)

- In thermocycler, incubate at 16°C for 90 min, then heat-kill at 65°C for 10 min. After the heat-kill, cool the solution at 2°C per 90 seconds until it reaches room temperature. Store at 4˚C (program “DDRAD_LIGIT" in “SAARMAN” folder).

5b. Clean to remove unincorporated adaptors or other fragments less than 100 bp with AmpureXP magnetic beads. Make sure the AMPure XP is at room temp and well mixed before starting!

1. Add **75 µL* AMPure XP** to each 50 µL ligated product
   a. Pipette up & down 10 times as you are adding the beads to the well
   b. NOTE: *1.5X the volume of the ligated product volume, adjust volume based on PCR volume and *the SPRI bead batch.*
2. Incubate at room temperature for **10 min**
3. Place reactions in magnetic plate for **2 min**
4. Aspirate the cleared solution and discard
5. Ethanol wash: Dispense 200 µL of fresh 70% ethanol to each well of the reaction plate
   a. Incubate for 30 seconds at room temperature
   b. Aspirate out the ethanol and discard
6. Repeat the last step one more time for a total of 2 ethanol washes.
   a. Make sure you get all of the ethanol out the second time, using a smaller pipette if needed.
   b. Let beads dry for 1-2 minutes
   c. Making sure you don’t let it get too dry (cracking-earth look = too dry)
7. Off the magnet plate, add 44 µL of µLtraPure H2O.
   a. Pipette up & down 10 times to completely mix the beads (that have the DNA on them)
8. On the magnetic plate, allow separation for 1 minute, carefully transfer 40 µL (now with the DNA) to a new plate/tube. Take care to leave the beads behind.

6. PCR of Ligated Fragments. For each plate of samples, in 40 µL reactions split into duplicate 20 µL reactions. Each plate will have its own mastermix, so make 2 tubes of mastermix.

X1 HF Phusion PCR		**X110 samples** (96 + 14 slop = 110) in 2 tubes of mastermix:	
1 µL	Ill_pcr1* primer (**10 µM**)	110 + 110	µL
1 µL	Ill_pcr2* primer (**10 µM**)	110 + 110	µL
4 µL	5X Phusion HF buffer	440 + 440	µL
0.4 µL	dNTPS (10 mM each)	44 + 44	µL
0.2 µL	HF Phusion Taq polymerase	22 + 22	µL
8.4 µL	ULtraPure H2O to volume (15 uL)	840 + 840	µL
15 µL	(duplicates for each sample)	1650 + 1650	µL (2 tubes of mastermix)

(15 µL mm per well, plus 5 µL ligated sample, in duplicates of 20 µL reactions)

- Remember this is in duplicates, so 1 plate from step 5 splits to 2 plates with reaction volumes of 20 uL each
- Add 15 µL master mix per well, you will need two tubes of mastermix to have enough.
- Add 5 µL DNA (ligations) to each well
- Make sure to mix well and spin down before proceeding.
- Run the program “DDRAD_PCR_65” in the “SAARMAN” folder for 30 cycles with the following program:
  - 98◦C for 30s
  - **15-20* cycles of:** 98◦C for 10s, 65◦C for 30s, 72◦C for 30s
  - final extension at 72◦C for 10 min.

  * For the alkali bee project, 12 cycles was too few, 30 cycles was OK but lots of PCR duplicates (both 60 ˚C or 70 ˚C annealing worked when we tested it, so we are going with 65 ˚C).

7. Clean each PCR individually with AmpureXP magnetic beads.

Make sure the AMPure XP is at room temp and well mixed before starting!

9. Combine the two PCR reactions for a total volume of 40 µL
10. Add **60 µL* AMPure XP** to each 40 µL PCR product.
    a. Pipette up & down 10 times as you are adding the beads to the well
    b. NOTE: *1.5X the volume of the PCR liquid, adjust volume based on PCR volume
11. Incubate at room temperature for **10 min**
12. Place reactions in magnetic plate for **2 min**
13. Aspirate the cleared solution and discard
14. Ethanol wash: Dispense 200 µL of fresh 70% ethanol to each well of the reaction plate
    a. incubate for 30 seconds at room temperature
    b. Aspirate out the ethanol and discard
15. Repeat the last step one more time for a total of 2 ethanol washes.
    a. Make sure you get all of the ethanol out the second time, using a smaller pipette if needed.
    b. Let beads dry for 1-2 minutes
    c. Making sure you don't let it get too dry (cracking-earth look = too dry)
16. Off the magnet plate, add 27 µL of µLtraPure H2O.
    a. Pipette up & down 10 times to completely mix the beads (that have the DNA on them)
17. On the magnetic plate, allow separation for 1 minute, carefully transfer 25 µL (now with the DNA) to a new plate/tube. Take care to leave the beads behind.

8. Quantify PCR products with Qubit or Plate-Reader (Quant-it Picogreen), see Appendix VII.

Use Qubit or Plate-Reader quantities from step above. This information is used to estimate what volume you want to pool from each sample/well in order to standardize PCR product quantity for each set of 24 samples using the equation:

$$C1 \times V1 = C2 \times V2$$

9. Pool individually barcoded samples with Amicon Ultra 30KDA centrifugal filter columns.

In this step, combine PCR product from 24 samples (individually barcoded completed PCR reactions) into a single 2 mL Lo-Bind tubes (come with the Amicon Ultra kit). We use Amicon Ultra 30KDA for this step so that we can standardize PCR product quantity and obtain a **final volume of 30µL** for each tube submitted for BluePippin size selection. To calculate what volume to add from each sample, use the DNA quantity from above and the equation C1 x V1 = C2 x V2.

1. Simply pipette all the volume from each sample you want to pool into one Amicon spin-column
2. Spin at 14,000 xg for 10 min,
3. Add 300 uL 1X buffer AE (Qiagen elution buffer) to the column, spin at 14,000 xg for 10 min
4. Flip column over a new tube, spin at 1,000 xg for 2 min to collect ~25-30 uL, you can top to 30 µL if you suspect a smaller volume.

10. Submit to CIB sequencing aaron.thomas@usu.edu

1. At this step, for a plate of 96 individuals, you should have **four separate LoBind tubes** provided in the Amicon Ultra kit, each containing PCR from 24 pooled individuals, which will be loaded onto the BluePippin separately. You can estimate concentrations using the ng added in the step before (example provided in the TEMPLATE ddRAD library prep spreadsheet), or you can Qubit in the Saarman lab to quantify.

2. **Prepare forms at https://usu.co1.qualtrics.com/jfe/form/SV_2tM3Yxd2IxK9Onk. You will need to complete this form twice, for (a) size selection, and (b) Illumina sequencing.** For each time you fill out the form you will also need to provide directions and upload a file with the sample information, with the templates provided.

    a. **Blue Pippin Size selection: https://usu.co1.qualtrics.com/jfe/form/SV_2tM3Yxd2IxK9Onk**

- Contact name. Your name.
- PI. Norah Saarman
- Contact email address. Your email address.
- Phone number. Your phone number.
- Department. Biology.
- On campus or off campus. On campus.
- Project Name: e.g. “Culex mosquito ddRAD”, “Alkali bee ddRAD”, etc.
- How will you pay? OCC or index number.
- OCC or index number: Discuss with Norah ahead of time.
- Service requested: Size selection (Blue Pippin)
- How many samples? 4*

  **NOTE* For a typical plate of 96 samples, you should have 4 samples (each with 24 individually barcoded specimens/libraries per sample). If you have an incomplete plate or fewer pools, adjust accordingly.*

- Do you want your samples analyzed on the TapeStation before and/or after size selection?

  ◉ TapeStation before size selection, please send me the data first to verify before proceeding with size selection

- Please enter any notes about the request.

  “This is a ddRAD library, so many of the DNA fragments should be much smaller than our target size range. We would like you to select with Blue Pippin for 460-660 bp*. Please run TapeStation before and after size selection, and send results to me before proceeding with size selection. If all goes well, we would also like TapeStation run after size selection.”

  **NOTE: Make sure the size range you request is specific to your project, and that you have accounted for the 103 bp added during the adapter ligation and PCR. For alkali bees, we have used 460-660 bp, which equates to selection of ~350-550 bp insert sizes. For Culex mosquitoes, we have used >500 bp, which equates to selection of ~400-1500 bp insert sizes to capture mtDNA and Wolbachia.*

- Upload the sample information: The template for this sample information is available at https://caas.usu.edu/biosystems/files/blue-pippin-submission.xlsx**.Make sure to check the box At the bottom of the form, you should have **four samples listed.** Type of DNA = “ddRAD library”. Buffer = “1X AE (Qiagen)”. Concentration should be calculated based on total ng per sample included in the pool, example available in the

TEMPLATE ddRAD library prep spreadsheet. Total volume = 25-30 µL. Size range desired = 460-660 bp*

Please see the example below:

	Sample Name	Type of DNA (PCR, seq library, etc.)	buffer	Sample concentration (ng/µl)	Total volume	size range desired
1	bee_ddRAD_3.1	ddRAD library	1X AE (Qiagen)	651	25-30 µL	460-660 bp*
2	bee_ddRAD_3.2	ddRAD library	1X AE (Qiagen)	651	25-30 µL	460-660 bp*
3	bee_ddRAD_3.3	ddRAD library	1X AE (Qiagen)	761	25-30 µL	460-660 bp*
4	Cx_ddRAD_1.4	ddRAD library	1X AE (Qiagen)	543	25-30 µL	>500 bp*

**NOTE: Make sure the size range you request is specific to your project, and that you have accounted for the 103 bp added during the adapter ligation and PCR. For alkali bees, we have used 460-660 bp, which equates to selection of ~350-550 bp insert sizes. For Culex mosquitoes, we have used >500 bp, which equates to selection of ~400-1500 bp insert sizes to capture mtDNA and Wolbachia.*
***NOTE: There is also a .pdf version out there, but I strongly recommend you use the excel version for ease of use and to reduce redundancy with the Qualtrics survey.*

b. **Illumina sequencing: https://usu.co1.qualtrics.com/jfe/form/SV_2tM3Yxd2lxK9Onk**

- Contact name. Your name.
- PI. Norah Saarman
- Contact email address. Your email address.
- Phone number. Your phone number.
- Department. Biology.
- On campus or off campus. On campus.
- Project Name: e.g. “Culex mosquito ddRAD”, “Alkali bee ddRAD”, etc.
- How will you pay? OCC or index number.
- OCC or index number: Discuss with Norah ahead of time.
- Service requested: Illumina Sequencing.
- Sequencing type: NextSeq 2000 P1 300 cycles
- How many runs/samples? 1*

  **NOTE* For a typical plate of 96 samples, you should have 1 sample at this step.*

- Are the sequencing libraries already prepared?
  1. Yes, only sequencing required, samples to be demultiplexed
  2. Yes, only sequencing required, no demultiplexing required
  3. No, library will be required in addition to sequencing

- Please enter any notes about the request.

  "This is a ddRAD library. For sequencing we would like NextSeq 2000 P1 (300 cycles), spiked with 15% PhiX, 300 bp reads from the forward end.* We would like all four of the samples pooled together with equal quantities based on your QC. These samples are internally barcoded, and we will do our own demultiplexing."

  **NOTE* Make sure the sequencing directions match other previous runs for your project. For example, for alkali bees, you would want to ask for "300 bp reads from the forward end". For Culex mosquitoes, you would want to ask for "300 bp reads from the forward end" to match what Emily has done previously. Another option for the 300 cycle runs is to request "150 bp paired-end reads".*

- Upload the sample information: While filling out the form for illumina sequencing, you will need to upload a sample submission sheet following the template, found at https://caas.usu.edu/biosystems/files/illumina-sequencing-submission.xlsx.** Here you should have **just one sample listed**, and you need to specify that you want the four tubes from the Blue Pippin step pooled for a single lane of sequencing (see previous bullet point above for an example of the words you might use). Leave the i7 index and i5 index fields blank.

  Please see the example below:

Name		Norah Saarman - bee ddRAD plate 2				
Submission date		15-Apr-24				
	Sample ID	Sample name	i7 index name	i7 index sequence	i5 index name	i5 index sequence
1	plate2	bee_ddRAD_plate2				

  ***NOTE: There is also a .pdf version out there, but I strongly recommend you use the excel version for ease of use and to reduce redundancy with the Qualtrics survey.*

3. **Submit to CIB sequencing aaron.thomas@usu.edu** with advance notice.

Appendix I: Diagram of oligos and adapters

Diagram of oligos and adapters

1. Double digest with EcoRI and MspI

EcoRI cut pattern:

```
5´... G▼A A T T C ...3´
3´... C T T A A▲G ...5´
```

MspI cut pattern:

```
5´...C▼C G G ...3´
3´...G G C▲C ...5´
```

Resulting fragments with cutsite:

```
5' AATTCNNNNNNNNNNC 3'
      3' GNNNNNNNNNNGGC 5'
```

2. **Ligate adaptors.** Adaptors include adaptor sequence, barcode, cutsite (EcoRI, Msp1), protector base, and phosphorothioate modification [*].

P1 Adaptor with barcode and EcoRI overhang — P2 adaptor with MspI overhang

```
 5' CTCTTTCCCTACACGACGCTCTTCCGATCTATCAGACACGCAATTCNNNNNNNNNNNNNCCGCAGATCGGAAGAGCTCGTATGCCGTCTTCTGCTTG 3'
3' TGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGATAGTCTGTGCGTTAAGNNNNNNNNNNNNNGGCGTCTAGCCTTCTCGAGCATACGGCAGAAGAC*G 5'
```

3. **PCR.** Primers Ill_pcr1* and Ill_pcr2* amplify barcoded fragments with correct cutsites and adaptors:

NNNNNNNNN = Insert (i.e. the actual data)

P1 adapter primer (Ill_pcr1*)

```
5' A*ATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATC*T 3' -- >
```

P1 Adaptor with barcode and EcoRI overhang — P2 adaptor with MspI overhang

```
 5' CTCTTTCCCTACACGACGCTCTTCCGATCTATCAGACACGCAATTCNNNNNNNNNNNNCCGCAGATCGGAAGATCTCGTATGCCGTCTTCTGCTTG 3'
3' TGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGATAGTCTGTGCGTTAAGNNNNNNNNNNNNGGCGTCTAGCCTTCTAGAGCATACGGCAGAAGAC*G 5'

                                   < --  3'               TAGAGCATACGGCAGAAGACGAAC 5'
```

P2 adaptor primer (Ill_pcr2_UMI4,5,6)

3. **Size selection.** Fragment loaded into the Blue Pippin for size selection:

```
5'A*ATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTATCAGACACGCAATTCNNNNNNNNNNNNNCCGCAGATCGGAAGAGCTCGTATGCCGTCTTCTGCTTG 3'
5' T*TACTATGCCGCTGGTGGCTCTAGATGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGATAGTCTGTGCGTTAAGNNNNNNNNNNNNNGGCGTCTAGCCTTCTCGAGCATACGGCAGAAGACGAACNNNN
```

Oligos and adaptors final total length in base pairs (bp):

5' end oligos and adapters: 69 bp
3' end oligos: 34 bp

Total: 103 bp

Appendix II: List of oligos and adapters

EcoRI adapters with 384 barcodes. Already annealed, from Zach Gompert, October - 2023

Oligos planned to order April 25, 2025

Name	OligoSequence
MspI_P2.1(T)[5PHO]_2025	5’ /5Phos/CGCAGATCGGAAGATCTCGTATGCCGTCTTCTGCTTG 3’
MspI_P2.2(A)_2025	5’ GCAGAAGACGGCATACGAGATCTTCCGATCTG 3’
Ill_pcr1_2025	5’ AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT 3’
Ill_pcr2_2025	5’ CAAGCAGAAGACGGCATACGAG 3’

*****_Note: Matches both existing plate 3 adapters (ligated fragments) and new adapters, by ending before the mismatch_

Oligos ordered in 2024

Name	OligoSequence
MspI_P2.1	5’ CGCAGATCGGAAGAGCTCGTATGCCGTCTTCTGCTTG 3’
MspI_P2.2*	5’ GCAGAAGACGGCATACGAGCTCTTCCGATCT*G 3’
Ill_pcr1*	5’ A*ATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATC*T 3’
Ill_pcr2*	5’ C*AAGCAGAAGACGGCATACGAGCTCTTCCGATCTGCG*G

PLANNING PHASE
Option 1 proposed by Aaron Thomas

Name	**OligoSequence**
MspI_P2.1	5’ /5Phos/CGCAGATC[Illumina primer + UMI]GGAAGAGCTCGTATGCCGTCTTCTGCTTG 3’
MspI_P2.2	matching…

Option 2 proposed by Aaron Thomas, more flexible and long term, try to incorporate biotinylated adaptors and amplicons for R21?

- Adaptor = sequence that matches the primer + UMI + 10 bp would be in the adaptor, make sure 5’ end of p1.2 has [PHO] and/or 5’ end of p2.2 has [PHO]
- Primer = P5 + index (illumina barcode) are on the primer, no bioinformatically processed barcode here… the demultiplex would be done by CIB
- Index hopping = a small number of molecules end up swapping index, somewhere around 0.5-1%

P2 adapter - with biotinylation bestRAD approach

Name	**OligoSequence**
MspI_P2.1	5’ /5Phos/CGCAGATCGGAAGAGCTCGTATGCCGTCTTCTGCTTG/3Biosg/ 3’
MspI_P2.2*	5’ G*CAGAAGACGGCATACGAGCTCTTCCGATCT*G 3’

Pcr1 and pcr2 primers

Name	**OligoSequence**
Ill_pcr1*	5’ A*ATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATC*T 3’
Ill_pcr2*	5’ C*AAGCAGAAGACGGCATACGAGCTCTTCCGATCTGCG*G

** Phosphorothioate [*] modification, PS bond substitutes a sulfur atom for a non-bridging oxygen in the phosphate backbone of an oligo, placing * between the last 3-5 nucleotides at the 5'- or 3'-end of the oligo inhibits exonuclease degradation.* ***NOTE:*** *Beware the [PHO] phosphorylation modification, a consequence of a minor spelling error in the Gompert protocol, which led Norah to order primers in error with [PHO] phosphorylation modification instead of [*].*

/3Biosg/ modification – Biotinylation modification so that we can use streptavidin coated beads to isolate desired fragments *IF/WHEN* we decide to use the “bestRAD” approach.

Phosphorothioate [*] - The ***correct*** *modification: https://eurofinsgenomics.com/en/products/dnarna-synthesis/mods/ confirms that [*] is phosphorothioate modification. “The phosphorothioate (PS) bond substitutes a sulfur atom for a non-bridging oxygen in the phosphate backbone of an oligo. This modification renders the internucleotide linkage resistant to nuclease degradation. Phosphorothioate bonds can be introduced between the last 3-5 nucleotides at the 5'- or 3'-end of the oligo to inhibit exonuclease degradation. Including phosphorothioate bonds throughout the entire oligo will help reduce attack by endonucleases as well.” - idt.com*

Phosphate [PHO] - The incorrect modification: eurofins.com “Phosphorylation allows the oligonucleotides to be used as a substrate for DNA ligase. 3' modifications can be used to block further extension by DNA. Terminal phosphates are also useful for enabling the ligation of two individual oligonucleotides together."

9/4/2024 note: Following PCR failure of August 2024, I recommend we use the new primers* with [*] phosphorothioate modification.

Appendix III: Real-Time PCR details

Run Setup

Run Information

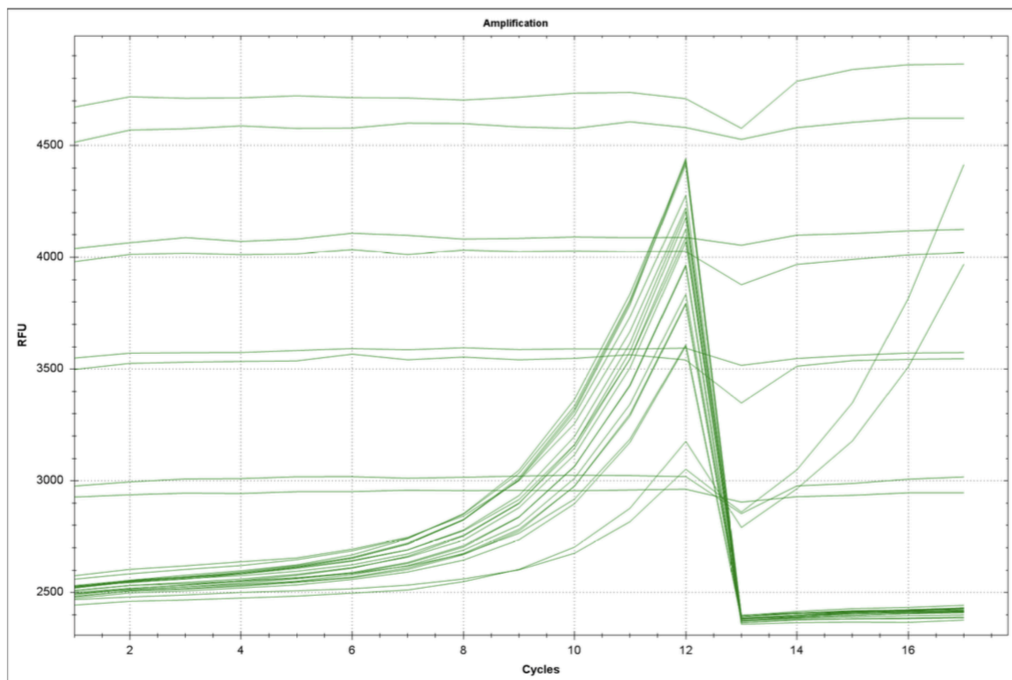
Run Date: 9/4/2013 11:55 AM
Run User: admin
Run Type: User-defined
ID:
Notes:
Sample Volume: 50
Temperature Control Mode: Calculated
Lid Temperature: 105
Base Serial Number: CT001211
Optical Head Serial Number: 785BR6657

Protocol

1: 98.0°C for 0:45
2: 98.0°C for 0:15
3: 65.0°C for 0:30
4: 72.0°C for 0:30
Plate Read
5: 72.0°C for 0:05
6: GOTO 2, 24 more times
7: 72.0°C for 1:00

Quantification

Step #: 4
Analysis Mode: Fluorophore
Cq Determination: Single Threshold
Baseline Method:
SYBR: Auto Calculated
Threshold Setting:
SYBR: None, Auto Calculated



Appendix IV: PEG-Sera-Mag™ (AmPure XP replacement) recipe

METHOD #1:

MATERIALS

- Sera-Mag SpeedBeads 15 ml, carboxylate-modified microparticles (ThermoScientific, cat.no. 6515-2105-050250, 470 €; see below for ordering in Germany)
- PEG-8000 powder (Promega)
- 5 M NaCl
- 0.5 M EDTA, pH 8.0
- 1 M Tris-HCl, pH 8.0
- Tween-20 (100%)
- TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0)

One order of Sera-Mag SpeedBeads is enough for 15 batches of 50 ml bead solution (~750 ml)
Total cost:
Total needed: ~14 mL per RAD run
~ 150 mL for all RAD runs.

PRINCIPLE

The final bead / buffer composition obtained from this protocol is as follows:

0.1% Sera-Mag beads
18% PEG-8000 (w/v)
1 M NaCl
10 mM Tris-HCl, pH 8.0
1 mM EDTA
0.05% Tween-20

Purification of PCR products using PEG and carboxylated beads is described by DeAngelis et al. 1995 in NAR (Solid-phase reversible immobilization for the isolation of PCR products.).

SIZE CUT-OFF

The size cut-off depends on the final concentration of PEG. This protocol recommends a concentration of 18% (w/v), which results in a size cut-off similar to the one found with commercial AMPure beads. If you want to experimentally infer the size cut-off, use DNA ladders from Fermentas (e.g. GeneRuler Ultra Low Range Ladder). Do not use NEB ladders!

STORAGE

Store the bead suspension in the fridge, protected from light. Experimentally test every few weeks if the beads are still working.

PROCEDURE

1| Add 9g PEG-8000 powder to a 50 ml falcon tube.

2| To the powder add 10 ml 5 M NaCl, 500 µl 1 M Tris-HCl and 100 µl 0.5 M EDTA. Fill up with water to ~ 49 ml. Shake the falcon tube until all PEG has dissolved. Add 27.5 µl Tween-20 and mix properly.

3| Resuspend stock solution of Sera-Mag beads by shaking. Transfer 1 ml bead suspension to a 2 ml Eppendorf tube and pellet the beads in a magnetic rack. Remove storage buffer and wash beads twice with 1 ml TE. Fully resuspend the beads in 1 ml TE.

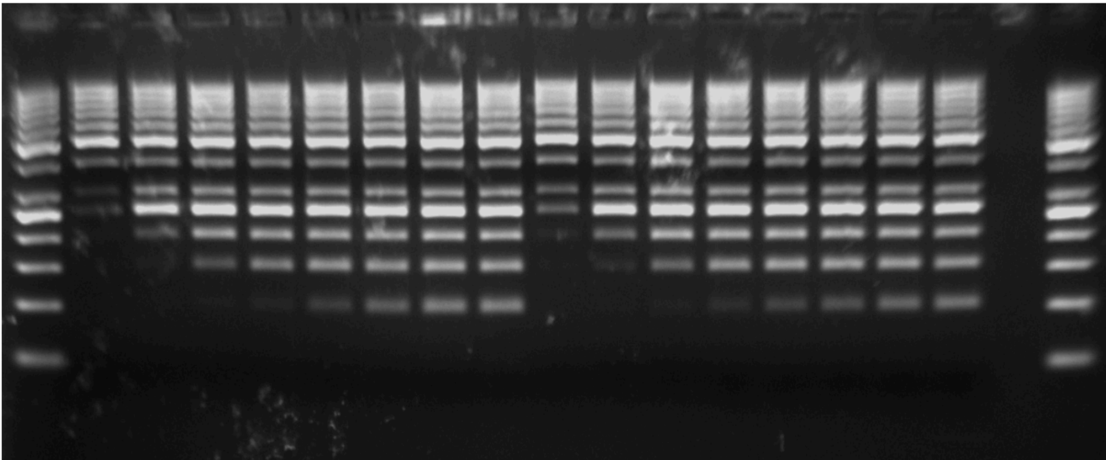
4| Add the bead suspension to the falcon tube and mix immediately. Wrap falcon tube in aluminium foil and store in fridge.

RESULTS

Aliquots of 1 µg 50-bp ladder (in 20 µl water) were purified using various ratios of bead/buffer suspension to sample, comparing the home-made SPRI suspension to the Agencourt AMPure XP kit. Results are shown in the figure.

|------------- home-made-----------------| |----------------AMPure------------------|

M 0.9 1.2 1.5 1.8 2.1 2.4 2.7 3.0 0.9 1.2 1.5 1.8 2.1 2.4 2.7 3.0 M



PEG-Sera-Mag™ (AmPure XP replacement) recipe

METHOD #2:

PROTOCOL FROM SARA WEINSTEIN

Solutions needed

TE - 100ml

What	Amount for 100ml
Tris pH8 1M	1 ml
EDTA 0.5M	200ul
Fill up with water, then autoclave	

NaCl-PEG solution

What	Amount for 250ml	Amount for 50ml
PEG 8000	45g	9g
NaCl	36.5g	7.3g
EDTA 0.5M	500ul	100ul
Tris pH8 1M	2.5ml	500ul
• Fill up with water • Mix carefully and wait until solution is clear (takes an hour or so, just wait) • filter with bottle-top filter		

SpeedBeads:

http://www.gelifesciences.com/webapp/wcs/stores/servlet/ProductDisplay?categoryId=613051&catalogId=10101&productId=121280&storeId=11762&langId=-1

Find them here: D6 Beads and Antibodies and related

Preparation

You need 1ml of the Sera-Mag Magnetic SpeedBeads stock solution for 50ml SPRI beads.

1. Pipet 1 ml of the bead stock solution into a 2 ml tube, and place it on the magnet (if you prepare more than 1 ml, use several tubes)
2. Let beads bind to the magnet for 5min, then discard the supernatant.
3. Wash beads twice with 1ml TE (invert tube so that beads completely resuspend, then place back on the magnet)
4. Remove tube from magnet, add 1ml TE and mix
5. Carefully add beads (with the TE they are in) into the NaCl-PEG solution
6. Mix very carefully
7. Store beads in the dark at 4C

Testing the beads

Compare to commercial AMPure XP beads

To clean/load 1 µg ladder in 50 uL volume:

- Dilute the Mix ladder with water, enough for 2 strip tubes:

X1		X17 (16 + 1 slop = 17)
2 µL	Low Molecular Weight Ladder @ 500 µg/ml*	34 µL
48 µL	Water	816 µL
50 µL	Dilute ladder for next step	850 µL

*NEB N3233S, or other ready-to-use 50 bp or 100 bp ladder

- Distribute the diluted ladder: 50 ul each well into 2 strip-tubes
- Add the following SRI beads/ AMPure bead amounts

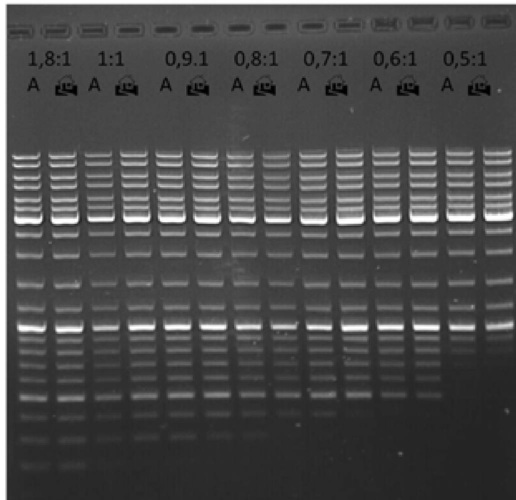
Ratio	**1.8 x : 1**	**1.5 x : 1**	**1 x : 1**	**0.9 x : 1**	**0.8 x : 1**	**0.7 x : 1**	**0.6 x : 1**	**0.5 x : 1**
Amount of ladder	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL
Amount of beads needed	90 µL	75 µL	50 µL	45 µL	40 µL	35 µL	30 µL	25 µL

To test very sticky beads… like we have been seeing lately:

Ratio	**1.5 x : 1**	**1 x : 1**	**0.8 x : 1**	**0.5 x : 1**	**0.4 x : 1**	**0.3 x : 1**	**0.2 x : 1**	**0.1 x : 1**
Amount of ladder	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL
Amount of beads needed	75 µL	50 µL	40 µL	25 µL	20 µL	15 µL	10 µL	5 µL

- Mix very well by pipetting
- Incubate at room temperature for **10 min**
- Place reactions in magnetic plate for **2 min**
- Aspirate the cleared solution and discard
- Ethanol wash: Dispense 200 µL of fresh 70% ethanol to each well of the reaction plate
    - incubate for 30 seconds at room temperature
    - Aspirate out the ethanol and discard
- Repeat the last step one more time for a total of 2 ethanol washes.
    - Make sure you get all of the ethanol out the second time, using a smaller pipette if needed.
    - Let beads dry for **1-2** minutes, making sure you don't let it get too dry (cracking-earth look is too much).
- Off the magnet plate, add 30 µL of µLtraPure H2O.
    - Pipette up & down 10 times to completely mix the beads (that have the DNA on them)
- On the magnetic plate, allow separation for **1 minute**, carefully transfer 25 µL (now with the DNA) to a new plate/tube. Take care to leave the beads behind.
- Load 8 ul of the eluted DNA with 2 ul loading dye
- Load 1 lane of the ladder as shipped following manufacturers recommendations as reference
- Run gel without stain, then stain by placing in a buffer with the same amount of stain you would have used after running the gel rather than our usual protocol (this helps make the bands more crisp, see NEB website on stains and low molecular weight ladder for more info.)

Gel should like this, but include a lane with fresh ladder for reference, and label* the ladder please!



NOTE: * It is really important that we know where the 100 bp band is so that we know what bead concentration to use before PCR amplification!!!

Aliquoting the beads

If you prepare >50ml of beads: aliquot them into 50ml Falcons.

Important: beads sink to the bottom rather quickly, so you need to ensure that all aliquots get the same amount of beads

a) continuous (proper) mixing of the bigger bottle while aliquoting

b=better) ensure all aliquots get a 'top of the bottle' and a 'bottom of the bottle' scoop while still trying to keep the whole bottle mixed well.

Credit and further reading:

Rohland N, Reich D. Cost-effective, high-throughput DNA sequencing libraries for multiplexed target capture. Genome Research. 2012. Doi: 10.1101/gr.128124.111
DeAngelis MM, Wang DG, Hawkins TL: Solid-phase reversible immobilization for the isolation of PCR products. Nucleic Acids Res 1995, 23: 4742–4743.
Fisher S, et al.: A scalable, fully automated process for construction of sequence-ready human exome targeted capture libraries. Genome Biol 2011, 12:R1.
Lundin S, Stranneheim H, Pettersson E, Klevebring D, Lundeberg J: Increased throughput by parallelization of library preparation for massive sequencing. PLoS One 2010, 5:e10029.

Appendix V: BluePippin Cassette Kit Reference Chart

BluePippin™ Cassette Kit Reference Chart
Software v6.31 / v6.41 Cassette Definition Set 36

For Size Selection

Product Number	Size Range	Collection Times	Minimum Size Distribution as Expressed by (CV)	Electrophoresis Voltage	Gel Cassette Type	Marker Type	Marker
BDQ3010	100-250 bp	50-100 min	5%	100V DC	3% agarose	**Internal**	Q3
BDF2010	100-600 bp	50-100 min	8%	100V DC	2% agarose	**Internal**	V2
BEF2010	100-600 bp	50-100 min	8%	100V DC	2% agarose	External	M1
BDF1510	250 bp-1.5 kb	30-75 min	8%	150V DC	1.5% agarose	**Internal**	R2
BLF7510	1-6 kb	3-8 hr	10%	25V DC	0.75% agarose	External	S1
BLF7510	2-6 kb	1.4-2.4 hr	20%	Pulsed	0.75% agarose	External	S1
BLF7510	3-10 kb	2.3-3.1 hr	20%	Pulsed	0.75% agarose	External	S1
BUF7510	10-18 kb	2.9-3.7 hr	20%	Pulsed	0.75% agarose	External	U1
BMF7510	18-27 kb	3.8-4.3 hr	20%	Pulsed	0.75% agarose	External	T1
BHZ7510	50 kb target only	9-10 hr	20%	Pulsed	0.75% HR agarose	External	Z1

Appendix VI: Budget estimates per sample, calculated November 2023

Step	Unit	Product Number	Unit price	$/sample
DNA extraction	**250 samples**	---	**$1,156.77**	**$4.63**
DNeasy kit	500 samples, Qiagen Kit + ProtK	Qiagen Cat. ID 19133, 69506	$2,029.83	$4.05
Tips p300	500 samples, TipOne 300 µL filter	USA Scientific	$95.10	$0.19
Tips p1000	500 samples, TipOne 1000 µL filter	USA Scientific	$113.40	$0.23
LoBind Tubes	500 samples, bag of 250 x 2	Eppendorf	$75.4	$0.15
Library Prep	**In libraries of 16-96**	---	**$580 (Pico)** $743 (Qubit)	**$5.80 (Pico)** $7.43 (Qubit)
T4 ligation of adapters	100 samples .2 µL each = 20 µL (400,000 units)	NEB M0202S	$68.00	$0.68
MluC-I	500 samples .2 µL each = 100 µL (1000 units)	NEB R0538S	$72.00	$0.15
Nla-III	250 samples .2 µL each = 50 µL (500 units)	NEB R0125S	$75.00	$0.30
HF PHUSION Taq	125 samples .4 µL each = 50 µL (100 units)	NEB M0530S	$120	$0.96
Blue Pippin	800 samples (10 Cassettes – 16/lane)	Sage BDF2010	$565	$0.71
BioAnalyzer	960 samples (Libraries of 96, 10 chips, Agilent HS DNA Kit)	Agilent 5067-4626	$790	$0.79
Quant-iT PicoGreen dsDNA Reagent	900 samples (2000 assays)	Invitrogen P7589	$534	$0.59
~~Qubit (Invitrogen)~~	~~200 samples (500 kit)~~	~~Q32853, Q32856~~	~~$344 +$101~~	~~$2.22~~
PCR plates	200 samples (Plates x 10)		$109.30	$0.55
Tips	TipOne Sterile filter tips x 15	USA-scientific	$60.52 x 1.5	$0.91
LoBind Tubes Etc.	500 samples (250 tubes, 1 tube per 2 samples, x2 for wiggle room)	Eppendorf	$37.70 x 2	$0.16
Sequencing	**In libraries of 16-96**	---	**$1,945 per library**	**$15.89-$60.78**
YCGA ($54,000 budget = max 27 lanes 1B reads)	32 samples per library, 31.25 million reads/sample, ~30X for 1 million fragments	NovaSeq 1 billion reads 2x150	$1,945	$60.78
USU CIB (NextSeq 2000 P1, $1,525, 100M reads, 2x150)	96 pooled per library, 1 million reads/sample, ~20X for 50K fragments	NextSeq 2000 P1 100 million reads 2x150	$1,525	$15.89

Total price for library prep before sequencing is roughly $5/sample for DNA extraction ($4.63/sample with DNeasy Kits), $6-$7/sample for library preparation ($5.80/sample with Picogreen Quant-it, $7.43/sample with Qubit). $10.50 to $12 per sample without sequencing costs.

For horseshoe crab:

Budget limit of $60,000.00 (over 2 years)
Target sample size (250 case + 250 control = 500 DNA extractions, 12 repeats for sequencing)

Step	Unit	Product Number	Unit price	Cost per sample	Cost for 500 samples
DNA extraction	2 x 250 sample kits	Qiagen Cat. ID 19133, 69506, etc.	$1,156.77 per 250	$4.63	$2,315.00
Library Prep	512 (32 sets of 16 individuals per size selection lane, then pool 2 for each sequencing library)	NEB M0202S, R0538S, R0125S, Sage BDF2010, Agilent 5067-4626, Invitrogen Q32853, etc.	$580 per 100 (Pico) or $743 per 100 (Qubit)	$5.80 (Pico) or $7.43 (Qubit)	$2,969.00 or $3,804.16
YCGA x 16 libraries (NovaSeq sequencing 1 billion reads 2x150)	512 total = 16 libraries x 32 samples per library NovaSeq 1 billion reads 2x150	NovaSeq gives you 31.25 million reads/ sample, ~30X for ~1 million fragments	$1,945 per 1 billion reads 2x150 X 16 libraries	$60.78	$31,120.00

This is the protocol I came up with to match the ~ 1 million fragments targeted
Reference genome https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_000517525.1
1.8 Gb size, GC content 33.5%
MluCI + NlaIII yields ~9,765,830 total A+B fragments
838570 fragments between 300 and 1000 bp*

*remember this means to size select for 436-1136 to accommodate the length of the adaptors added before size selection

Appendix VII: Quantify DNA with Plate-Reader (Quant-it Picogreen) Protocol in LSB 412

- Get permission to use the machine/reagent from Sara Weinstein (Quant-iT Pico Green dsDNA Assay Kit)

- Make standards from the stock solution (100 ng/µL) in a strip tube, one at full concentration, one at 1:10 dilution to safeguard against pipetting error.

	Standard	H2O to add:	Standard to add:	Target concentration (ng/µL)	Dilute 1:10 for next step…
A	SS_.25	20 uL	20 µL B	0.25 ng/µL	10 µL A + 90 µL H2O
B	SS_0.5	20 uL	20 µL C	0.5 ng/µL	10 µL B + 90 µL H2O
C	SS_1.0	20 uL	20 µL D	1 ng/µL	10 µL C + 90 µL H2O
D	SS_2.0	20 uL	20 µL E	2 ng/µL	10 µL D + 90 µL H2O
E	SS_4.0	40 uL	10 µL H	4 ng/µL	10 µL E + 90 µL H2O
F	SS_5.0	20 uL	20 µL G	5 ng/µL	10 µL F + 90 µL H2O
G	SS_10	20 uL	20 µL H	10 ng/µL	10 µL G + 90 µL H2O
H	SS_20	80 uL	20 µL stock solution*	20 ng/µL	10 µL H + 90 µL H2O

* stock solution of dsDNA standard is provided with the kit at 100 ng/µL

- For a plate of 96 plus 8 x 2 = 16 standards
  - First make 1X TE
  - Then mastermix (concentrated Quant-IT reagent should be thawed and spun down)
  - Then prep plates

X1 TE		X126 (96 + 14 slop + 16 standards = 126)
5 µL	20X TE	630 µL
95 µL	Sterile H2O	11,970 µL
		12.6 mL total volume

X1 Quant-IT Picogreen mastermix		X126
.25 µL	Concentrated Quant-IT reagent	31.5 µL
49.75 µL	1X TE (from above)	6,268.5 µL
		6.3 mL total volume

- Load sample and mastermix into black plates from Weinstein lab for plate reader:

  - For quant standards (only first two columns of black PCR plate):
    - Add 40 µL 1X TE to each well
    - 10 µL 1:10 dilute standard A-H from above
    - 50 µL mastermix (dilute reagent), pipette up and down to mix well

  - For each dsDNA sample:
    - Add 49 µL 1X TE to each well
    - 1 µL DNA
    - 50 µL mastermix (dilute reagent), pipette up and down to mix well

- **Run on plate reader Spectramax 2 using the "Nucleotides - Picogreen" protocol**
  - Get laptop from microscope area in 412 E
  - Plug in laptop to power and the gray USB cord attached to Picogreen machine
  - Turn on machine using switch on top right side of back of machine
  - Open up SoftMax Pro 7
  - Open up the Norah_ddRAD_9Aug2024
  - Connect machine in top left corner
  - Select Plate 1 with two blue column for Standards

- Add Standard Plate to machine - take clear lid off and add to purple plate adapter (found in cabinet under machine)
- Hit drawer button
- Select Plate 1 on computer and hit Read plate green button
- Add in Plate 2 with samples and hit read
- Once finished, hit drawer, shut off machine and put plastic cover back on
- In main file menu hit export, Unknowns no dilution and export as Both, output format
- Use flash drive to get file